proven to regulate gene expression, demethylation of newly expressed genes is often observed during differentiation. Our results suggest that genes transiently expressed during differentiation of a cell lineage may bear a stable residual demethylation pattern at later times. We have compared our results to other gene systems in which methylation has been studied, but found none that parallel ours in identifying a gene transiently expressed during differentiation (including the globin genes in which expression of fetal and adult forms of α and β globins does not necessarily occur in the same cell). However, analysis of methylation patterns in other systems may provide a general tool for sorting out complex precursor-product relationships in hemopoetic and other cell lineages.

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12. $CD4^-8^-$ thymocytes from B6 mice were prepared by removal of $CD8^+$ T cells on plastic petri dishes coated with AD4(15) [mouse immunoglobulin M (IgM) specific for murine CD8.2] (*13*). Nonadherent cells were coated with KJ16 (rat IgG specific for murine T cell receptor Vβ8.1 and 8.2 regions) (*14*), GK1.5 (rat IgG specific for murine CD4) (*15*), and 2.43 (rat IgG specific for CD8.2) (*16*) and incubated on petri dishes coated with goat antibody to rat IgG (Jackson ImmunoResearch Laboratories Inc.). Nonadherent cells were collected and passed two more times over goat antibody to rat IgG–coated plates. Purified $CD4^-8^-$ cells were analyzed by flow cytometry (EPICS C, Coulter Electronics), with 2.43 and GK1.5 as primary staining reagents and fluroscein isothiocyanate (FITC)–labeled RG7/9.1 (mouse antibody to rat κ chain, gift of T. A. Springer) as the secondary reagent. By this analysis 90% of the purified cells were $CD4^-8^-$. The remaining 10% expressed only low density surface CD4, CD8, or both. High density J11d (gift of J. Sprent) was observed on 97% of purified double negative cells, and low density T3 (145-2C11, gift of J. Bluestone) was observed on 6% of these cells. The $CD4^+8^+$ thymocytes were isolated from newborn B6 mice; 97% of these cells stained with both GK1.5 and 2.43. The $CD8^+$ T cells (94% pure) were isolated as described (7). The $CD4^+$ T cells from B6 lymph node were isolated by passage over nylon wool columns and nonadherence to AD4 (*15*) coated plates; 97% of these T cells expressed surface CD4.
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Inositol 1,3,4,5-Tetrakisphosphate Induces Ca^{2+} Sequestration in Rat Liver Cells

TIMOTHY D. HILL, NICHOLAS M. DEAN, ALTON L. BOYNTON*

Inositol 1,4,5-trisphosphate [I(1,4,5)P_3] is a second messenger generated along with diacylglycerol upon the binding of various physiological agents with their cell surface receptors. I(1,4,5)P_3 mobilizes Ca^{2+} from intracellular storage sites through a receptor-coupled mechanism, and the subsequent increased intracellular free calcium ion concentration ($[Ca^{2+}]_i$) activates a multitude of cellular responses. Electropermeabilized neoplastic rat liver epithelial (261B) cells were used to study Ca^{2+} sequestration, a process that reverses the elevated $[Ca^{2+}]_i$ to resting levels and replenishes intracellular Ca^{2+} pools. Although I(1,4,5)P_3-mobilized Ca^{2+} is readily sequestered into storage pools by the action of Ca^{2+}–adenosine triphosphatases, Ca^{2+} mobilized by addition of the nonmetabolized inositol trisphosphate isomer I(2,4,5)P_3 is not sequestered, suggesting that metabolism is necessary to eliminate the stimulus for Ca^{2+} release. Several inositol phosphate compounds were examined for their ability to lower the buffer $[Ca^{2+}]$ to determine if a specific I(1,4,5)P_3 metabolite might be involved in stimulating Ca^{2+} sequestration; of these, I(1,3,4,5)P_4 alone was found to induce Ca^{2+} sequestration, demonstrating a physiological role for this inositol trisphosphate metabolite.

THE SECOND MESSENGER ROLE OF I(1,4,5)P_3 as a Ca^{2+} mobilizing agent is well characterized (*1*). Transient and prolonged alterations in $[Ca^{2+}]_i$ in response to the liberation of I(1,4,5)P_3 are known to stimulate such diverse physiological functions as secretion, egg fertilization, and cell proliferation (*2*). Many enzymes are activated by a rise in $[Ca^{2+}]_i$ either directly by free Ca^{2+} or through association with the calcium-binding protein calmodulin (*3*). Moreover, a number of calcium-binding proteins exist that might participate in important regulatory mechanisms when complexed with Ca^{2+}, for which a functional role has yet to be assigned (*4*). Although the release of intracellularly stored Ca^{2+} induced by I(1,4,5)P_3 appears to be receptor mediated (*5*) and in tight association with calmodulin (*6*), the mechanism for initiating a reversal of high $[Ca^{2+}]_i$ after the Ca^{2+} mobilization response, and thereby removing the stimulatory signal generated by Ca^{2+}, has not been identified. To study Ca^{2+} sequestration we used an electropermeabilized neoplastic rat liver epithelial (261B) cell system, in which changes in buffer $[Ca^{2+}]$ were monitored with the fluorescent indicator fura-2, and we found that inositol 1,3,4,5-tetrakisphosphate [I(1,3,4,5)P_4] induces Ca^{2+} sequestration.

When 2 μ*M* I(1,4,5)P_3 (a maximal dose) was added to permeable 261B cells, there was a rapid release of Ca^{2+} into the buffer, followed by a sequestration phase that reduced the buffer $[Ca^{2+}]$ to starting levels (Fig. 1A). In contrast, Ca^{2+} mobilized by 2 μ*M* I(2,4,5)P_3 was not sequestered by intracellular membranes (Fig. 1B). This finding could be the result of receptor de-

Cancer Research Center of Hawaii, University of Hawaii, Honolulu, HI 96813.

*To whom correspondence should be addressed.

Table 1. Effect of inositol phosphate compounds on Ca^{2+} sequestration. Inositol phosphate (IP) compounds were added 30 s before I(2,4,5)P_3, and the amount of Ca^{2+} released was determined. The amount of Ca^{2+} sequestered after a 15-min incubation period was determined on the same permeabilized cell samples. Experiments were repeated ten times with similar results.

Inositol phosphate (2 μ*M*)	Ca^{2+} released by 2 μ*M* I(2,4,5)P_3 (n*M*)	Ca^{2+} sequestered in response to IP (n*M*)
I(1)P_1	289	0
I(2)P_1	322	0
I cyclic(1,2)P_1	278	0
I(1,4)P_2	312	0
I(1,3,4)P_3	354	0
I(1,3,4,5)P_4	368	368
I(1,4,5,6)P_4	397	0
I(*w*,*x*,*y*,*z*)P_4	264	0
I(1,3,4,5,6)P_5	292	0
I(1,2,3,4,5,6)P_6	388	0

sensitization or the failure of cells to metabolize the Ca^{2+} mobilizing agent. Because repeated release and uptake of Ca^{2+} by multiple additions of $I(1,4,5)P_3$ occurs in 261B cells (*7*), we dismissed the possibility of receptor desensitization and hypothesized that Ca^{2+} sequestration involves the dissociation of $I(1,4,5)P_3$ from its receptor and metabolism either to $I(1,4)P_2$ by a 5-phosphomonoesterase (*8*) or to $I(1,3,4,5)P_4$ by a Ca^{2+}- and calmodulin-dependent inositol 3-kinase (*9*) to remove the Ca^{2+} release stimulus. Studies of $I(1,4,5)P_3$ metabolism in 261B cells show that after 2 min of incubation more than 70% of starting $[^3H]I(1,4,5)P_3$ was converted predominantly to $[^3H]I(1,4)P_2$ and $[^3H]I(1,3,4,5)P_4$, while $I(2,4,5)P_3$, a poor substrate for the inositol 3-kinase (*10*), remained unmetabolized throughout the 16-min incubation period (Fig. 2C). Levels of $[^3H]I(1,3,4,5)P_4$ reached a maximum at 2 min and gradually declined for the remainder of the experiment (Fig. 2A); this temporal pattern parallels the Ca^{2+} release and uptake response (Fig. 1A), suggesting that this metabolite of $I(1,4,5)P_3$ might function to induce Ca^{2+} sequestration.

In further studies, we observed that addition of $I(1,3,4,5)P_4$ just before, but not a few minutes after, $I(2,4,5)P_3$ addition (Fig. 1, C and D) caused a reversal of the high buffer $[Ca^{2+}]$ induced by $I(2,4,5)P_3$ in a dose-dependent manner (*11*). The specificity of $I(1,3,4,5)P_4$ in inducing sequestration of released Ca^{2+} was indicated by the fact that $I(1,4,5,6)P_4$, randomly labeled $I(w,x,y,z)P_4$, $I(1,3,4,5,6)P_5$, and $I(1,2,3,4,5,6)P_6$, as well as lower inositol phosphates including $I(1,3,4)P_3$, I(1)P, I(2)P, I cyclic$(1,2)P_1$, and $I(1,4)P_2$, could not mimic the effect of $I(1,3,4,5)P_4$ (Table 1).

In the presence of $I(2,4,5)P_3$, $[^3H]I(1,3,4,5)P_4$ was metabolized by 261B cells exclusively to $I(1,3,4)P_3$ (Fig. 2B) at a rate similar to that found in the $[^3H]I(1,4,5)P_3$ conversion studies, an indication that $I(2,4,5)P_3$ does not influence 5-phosphomonoesterase activity. In addition, the biological action of $I(1,3,4,5)P_4$ was not a result of stimulating the metabolism of $I(2,4,5)P_3$, because $[^3H]I(2,4,5)P_3$ was not metabolized by our 261B cells in the presence of $I(1,3,4,5)P_4$ (Fig. 2C).

Earlier studies indicated that the presence of $I(2,4,5)P_3$ was required to reveal a biological effect of $I(1,3,4,5)P_4$ (*10, 12*). In electropermeabilized rat liver cells, $I(2,4,5)P_3$ released Ca^{2+} without initiating subsequent Ca^{2+} sequestration, a result apparently due to the inability of 261B cells to metabolize $I(2,4,5)P_3$ because addition of the $I(1,4,5)P_3$ metabolite $I(1,3,4,5)P_4$ in combination with $I(2,4,5)P_3$ produced a biological effect. If $I(1,3,4,5)P_4$ was functioning alone in initiating sequestration, it should do so in the absence of $I(2,4,5)P_3$ and in a model system where $[Ca^{2+}]$ could be increased without stimulation of Ca^{2+} sequestration. We reasoned that if we could artificially increase $[Ca^{2+}]$ in the cell buffer by addition of $CaCl_2$, the Ca^{2+} storage

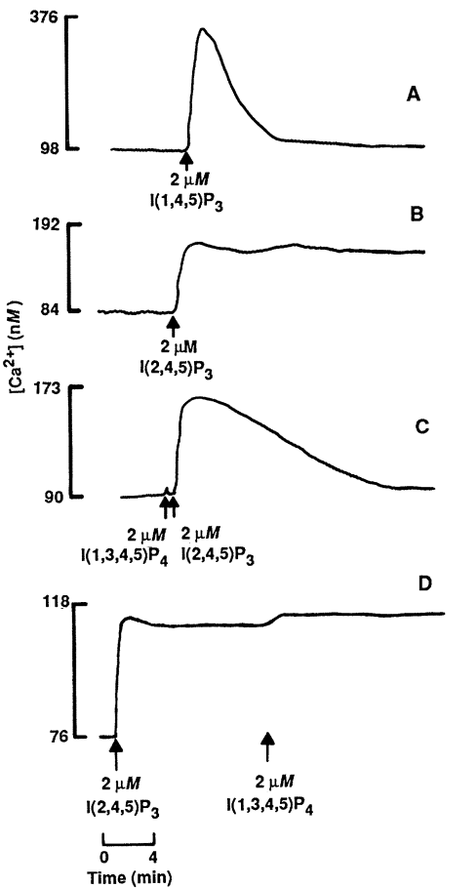

Fig. 1. Influence of $I(1,3,4,5)P_4$ on $I(2,4,5)P_3$-mobilized Ca^{2+} in permeable 261B rat liver epithelial cells monitored with the Ca^{2+} indicator fura-2. Tracings are the change in fura-2 fluorescence in response to (**A**) 2 μ*M* $I(1,4,5)P_3$; (**B**) 2 μ*M* $I(2,4,5)P_3$; (**C**) 2 μ*M* $I(1,3,4,5)P_4$ followed 30 s later with 2 μ*M* $I(2,4,5)P_3$; and (**D**) 2 μ*M* $I(2,4,5)P_3$ followed 5 min later with 2 μ*M* $I(1,3,4,5)P_4$. Arrows indicate the point of agent addition. The scale at the left represents the calculated change in $[Ca^{2+}]$ induced by the various treatments. The tracings are individual experiments representative of effects repeated 25 times. The cell response to $I(1,4,5)P_3$ was biphasic, composed of a rapid Ca^{2+} release and a slower sequestration phase; $I(2,4,5)P_3$ treatment mobilized Ca^{2+} but lacked the ability to initiate Ca^{2+} sequestration. Addition of $I(1,3,4,5)P_4$ (but not other inositol phosphates) with $I(2,4,5)P_3$ reversed the increased $[Ca^{2+}]$ induced by $I(2,4,5)P_3$. Cultured 261B neoplastic rat liver epithelial cells were grown to confluency (*14*). Cells were removed from culture dishes by mild treatment with trypsin. After two washes in cold phosphate-buffered saline (*p*H 7.4), cell samples (15×10^6 cells in 300 μl of high K^+ buffer: 140 m*M* KCl, 10 m*M* NaCl, 10 m*M* Hepes, 1 m*M* $MgCl_2$; *p*H 7.4) were electroporated with 20 discharges at 2 kV. Cells were washed twice with cold K^+ buffer and cooled to 4°C. Samples of permeabilized cells (5×10^6 cells in 0.5 ml of K^+ buffer) were incubated for 15 min at 30°C after addition of an ATP-regenerating system: 4 m*M* ATP, 10 m*M* creatine phosphate, and phosphocreatine kinase (250 mg/ml). Fura-2 (4 μ*M*) was added, and changes in fluorescence were monitored at 340 nm excitation and 500 nm emission at 30°C. The calcium concentration was calculated as described (*18*).

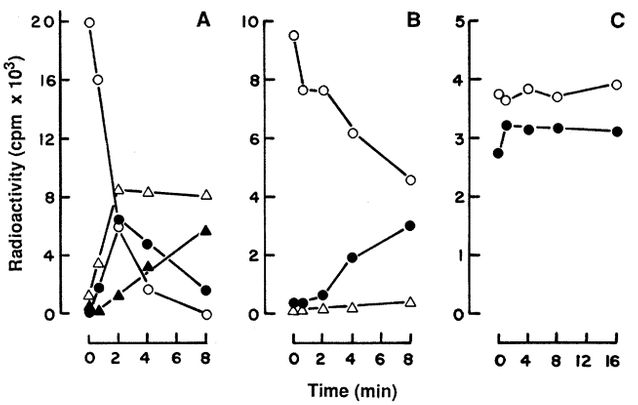

Fig. 2. Metabolism of $I(1,4,5)P_3$, $I(1,3,4,5)P_4$, and $I(2,4,5)P_3$ in permeable 261B cells. (**A**) $[^3H]I(1,4,5)P_3$ (2 μ*M*) was added to permeable cells and portions were removed at 0, 0.5, 2, 4, and 8 min. Metabolites detected included $[^3H]I(1,4,5)P_3$ (○), $[^3H]I(1,3,4,5)P_4$ (●), $[^3H]I(1,4)P_2$ (△), and $[^3H]I(1,3,4)P_3$ (▲). (**B**) $[^3H]I(1,3,4,5)P_4$ (2 μ*M*) was added to permeable cells and portions were removed at 0 and 0.5 min. Unlabeled $I(2,4,5)P_3$ (2 μ*M*) was then administered and portions were taken at 2, 4, and 8 min. Radiolabeled compounds detected were $[^3H]I(1,3,4,5)P_4$ (○) and $[^3H]I(1,3,4)P_3$ (●). A small amount of $[^3H]I(3,4)P_2$ (△) was formed. (**C**) $[^3H]I(2,4,5)P_3$ (2 μ*M*) was added alone (●) or 0.5 min after addition of 4 μ*M* unlabeled $I(1,3,4,5)P_4$ (○); portions were removed at 0, 0.5, 4, 8, and 16 min. Only unmetabolized $[^3H]I(2,4,5)P_3$ was detected. ^3H-labeled inositol phosphate (30 to 60 μl) was added to 2 ml of electropermeabilized cells in suspension (2×10^7 cells) under conditions identical to those used for the Ca^{2+} release experiments. $[^3H]I(1,4,5)P_3$, $[^3H]I(1,3,4,5)P_4$, and $[^3H]PIP_2$ (phosphatidyl inositol 4,5-bisphosphate) were obtained from Dupont Biotechnology Systems (Boston). $[^3H]I(2,4,5)P_3$ was prepared by alkaline hydrolysis of $[^3H]PIP_2$ (*19*). At each time point during the experiment duplicate portions (250 μl) of cell suspension were mixed with 100 μl of 25% trichloroacetic acid (TCA) to stop the reaction. After centrifugation (15,600*g*, 15 s) the supernatant was removed and TCA extracted by five washes with water-saturated diethyl ether. [^3H]Inositol phosphate metabolites were separated by high-performance liquid chromatography, and radioactivity was measured as described (*20*). Experiments were performed twice (in duplicate) with identical results.

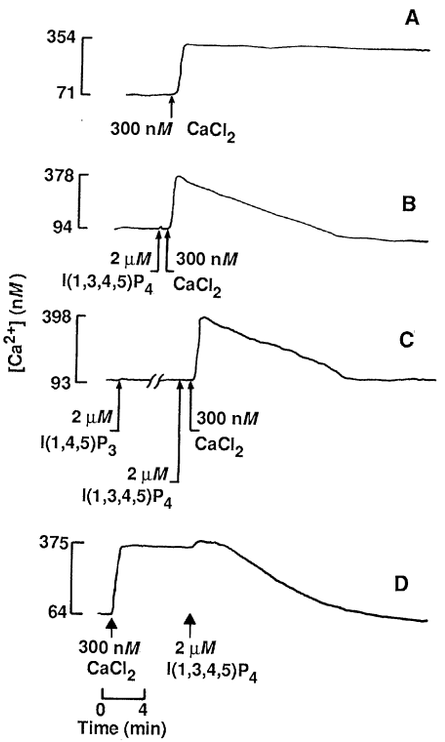

Fig. 3. Effect of $I(1,3,4,5)P_4$ on high levels of Ca^{2+} increased by exogenous addition of $CaCl_2$ to permeable 261B cells. Tracings of fura-2 fluorescence changes are shown for individual experiments representing results that were repeated 25 times. Cells were incubated for 15 min with excess $CaCl_2$ and tested as follows: (**A**) Permeable 261B cells received 300 n*M* $CaCl_2$. (**B**) $I(1,3,4,5)P_4$ (2 μ*M*) was added 30 s before 300 n*M* $CaCl_2$. (**C**) Cells were incubated for 15 min more with heparin (50 μg/ml) to inhibit Ca^{2+} release induced by $I(1,4,5)P_3$; then 2 μ*M* $I(1,3,4,5)P_4$ and 300 n*M* $CaCl_2$ were added. (**D**) $I(1,3,4,5)P_4$ (2 μ*M*) was added 5 min after 300 n*M* $CaCl_2$. Saturation of Ca^{2+} pools by incubation with excess $CaCl_2$ desensitized the sequestration mechanism to further addition of Ca^{2+}. $I(1,3,4,5)P_4$ initiated the cell sequestration of added Ca^{2+} in the absence of other inositol phosphates.

pools might reach a point at which the sequestration mechanism was downregulated. Initial addition of 300 n*M* Ca^{2+} to cell suspensions was sequestered into the Ca^{2+} storage pools and desensitized the Ca^{2+} uptake mechanism to further additions of Ca^{2+}. A second addition of Ca^{2+} increased $[Ca^{2+}]$ without subsequent sequestration (Fig. 3A). When a mixture of 2 μ*M* $I(1,3,4,5)P_4$ and 300 n*M* Ca^{2+} (Fig. 3B) was added to the cell suspension, $[Ca^{2+}]$ rapidly increased and levels returned to starting values over a 10- to 15-min period. Moreover, $I(1,3,4,5)P_3$ could initiate sequestration of Ca^{2+} even when added several minutes after $[Ca^{2+}]$ was elevated (Fig. 3D). We conclude that $I(1,3,4,5)P_4$ induced this phenomenon by itself and has no functional requirement for $I(2,4,5)P_3$. Further, heparin, which inhibits $I(1,4,5)P_3$ binding to high-affinity receptors (*13*) and inhibits the physiological receptor mediating $I(1,4,5)P_3$-induced Ca^{2+} release (*14*), had no effect on $I(1,3,4,5)P_4$-induced Ca^{2+} sequestration (Fig. 3C), indicating that $I(1,3,4,5)P_4$ interacts at a receptor site separate from the $I(1,4,5)P_3$ receptor site.

Our experiments demonstrate that $I(1,3,4,5)P_4$ stimulates permeable 261B cells to reduce Ca^{2+} levels raised by $I(2,4,5)P_3$ or $CaCl_2$ addition. These results suggest that not only is phosphorylation of the second messenger $I(1,4,5)P_3$ necessary to remove the stimulus for Ca^{2+} mobilization, but the newly formed metabolite $I(1,3,4,5)P_4$ serves as the inducer of Ca^{2+} sequestration by a receptor-mediated mechanism. The mechanism of $I(1,3,4,5)P_4$-induced Ca^{2+} sequestration is unknown but is not the result of $I(1,3,4,5)P_4$ displacing $I(2,4,5)P_3$ from its receptor, because $I(1,3,4,5)P_4$ binds to the $I(1,4,5)P_3$ receptor and to the $I(2,4,5)P_3$ receptor with a much lower affinity than $I(2,4,5)P_3$ (*13, 15*), and the $I(1,4,5)P_3$ receptor inhibitor heparin had no effect on $I(1,3,4,5)P_4$-induced Ca^{2+} sequestration.

Receptor occupancy by $I(2,4,5)P_3$ influences the action of $I(1,3,4,5)P_4$, possibly through a feedback inhibition mechanism. $I(1,3,4,5)P_4$ failed to reverse the increased buffer $[Ca^{2+}]$ when administered a few minutes after $I(2,4,5)P_3$ addition, although it was capable of reversing high Ca^{2+} levels when given after $CaCl_2$ and in the absence of $I(2,4,5)P_3$.

$I(1,3,4,5)P_4$ probably functions by interaction at specific binding receptors. High-affinity $I(1,3,4,5)P_4$ binding sites have recently been described in membranes of HL-60 cells (*16*) and various rat tissues (*17*). These specific binding sites might be physiological receptors coupled to the adenosine triphosphate (ATP)–dependent Ca^{2+} transport system responsible for sequestering Ca^{2+}. Therefore, it appears that the tightly regulated $[Ca^{2+}]_i$, which is increased by $I(1,4,5)P_3$-induced opening of Ca^{2+} channels, is reduced to resting levels by metabolic generation and action of $I(1,3,4,5)P_4$ and thus involves receptor-coupled mechanisms to both activate and deactivate intracellular membrane Ca^{2+} fluxes.

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